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ISOZYME SELECTIVE ARYLATION OF CYTOSOLIC GLUTATHIONE *S*-TRANSFERASE BY [^{14}C]BROMOBENZENE METABOLITES

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Abstract—[^{14}C]Bromobenzene was incubated with NADPH-fortified liver homogenates from phenobarbital-treated rats, after which the glutathione *S*-transferases were isolated from the incubation mixture. Glutathione *S*-transferase activity, with 1-chloro-2,4-dinitrobenzene as the substrate, in the homogenate was unchanged after incubation with bromobenzene. Radioactivity derived from the [^{14}C]bromobenzene remained associated with the cytosolic glutathione *S*-transferases after DE52 and Sephadex G-100 chromatography. Further purification of the cytosolic glutathione *S*-transferase by CM52 and hydroxylapatite chromatography showed that bromobenzene metabolites were bound to fractions containing glutathione *S*-transferase subunits 4, 5, and 1. The primary site of arylation appeared to be subunit 1, as indicated by autoradiography and hydroxylapatite chromatography. [^{14}C]Bromobenzene metabolites were not bound to microsomal glutathione *S*-transferases. These data show that hepatic cytosolic glutathione *S*-transferases, especially glutathione *S*-transferases 4-4/5-5, 3-4, and 1-1 may act as trapping or scavenger proteins for reactive metabolites and that this effect is not associated with a loss of catalytic activity

Bromobenzene causes centrilobular hepatic necrosis in rats, which is associated with the formation of reactive metabolites that covalently bind to liver macromolecules [1–4]. Bromobenzene-3,4-oxide, which is formed by the cytochrome P-450-dependent oxygenation of bromobenzene, has been tentatively identified as the reactive metabolite that binds to tissue proteins and depletes glutathione contents, although multiple reactive metabolites are formed [5, 6]. Hence, despite the association between metabolism and toxicity, a direct correlation between macromolecular covalent binding and toxicity of bromobenzene metabolites has not been established [7]. Glutathione *S*-transferases (EC 2.5.1.18) catalyze the conjugation of bromobenzene metabolites with glutathione, and this may serve to detoxify bromobenzene reactive intermediates [8]. Bromobenzene metabolites also become covalently bound to glutathione *S*-transferase 1-2 [9]. In the present study, the relationship between glutathione *S*-transferases and the covalent binding of bromobenzene metabolite was investigated *in vitro*. It was shown that bromobenzene metabolites become covalently bound to glutathione *S*-transferases, but that this arylation is not accompanied by a loss in catalytic activity with 1-chloro-2,4-dinitrobenzene (CDNB‡) as the substrate.

MATERIALS AND METHODS

[^{14}C]Bromobenzene (92 mCi/mmol; 86% pure) was purchased from Amersham (Arlington Heights, IL). 1-Chloro-2,4-dinitrobenzene was obtained from the Eastman Chemical Co. (Rochester, NY). CM-Cellulose (CM52) and DEAE-cellulose (DE52) were purchased from Whatman, Inc. (Clifton, NJ). Sephadex G-100 (superfine) and hydroxylapatite were obtained from Pharmacia Fine Chemicals (Piscataway, NJ) and Bio-Rad (Richmond, CA) respectively. NADPH was purchased from the Sigma Chemical Co. (St. Louis, MO).

Male Long-Evans rats (240–270 g) were given phenobarbital sodium (75 mg/kg) intraperitoneally once daily for 3 days, and the rats were killed by decapitation 24 hr after being given the last dose of phenobarbital. After perfusion of the liver *in situ* with 1.15% potassium chloride, the liver was removed and homogenized in 2 vol. of 0.01 M Tris–HCl buffer (pH 7.4) with a Teflon–glass homogenizer.

Twenty milliliters of rat liver homogenate (70–80 mg protein/ml) was incubated with 0.6 mM NADPH and 27 mM bromobenzene (including 0.054 mM [^{14}C]bromobenzene) at 37° for 30–40 min in closed flasks containing an atmosphere of air; control incubation mixtures lacked bromobenzene. The reaction was stopped by cooling the incubation flasks in an ice bath. The reaction mixture was centrifuged at 9,000 *g* for 30 min, and the supernatant fraction was centrifuged at 105,000 *g* for 60 min. The resultant supernatant was used as cytosol. The

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‡ Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DE52, DEAE-cellulose; CM52, CM-cellulose; SDS, sodium dodecyl sulfate; and GSH, glutathione. The several isozymes of the glutathione *S*-transferases are identified by their subunit structure [10].

105,000 *g* pellet was centrifuged twice more with 0.01 M Tris–HCl buffer (pH 7.4) at 105,000 *g* for 60 min and was stored at −20° until used.

For the separation of cytosolic glutathione *S*-transferases, the cytosol was applied to a DE52 column, which was eluted with 0.1 M Tris–HCl buffer (pH 8.0) and, after elution of nonadsorbed proteins was complete, with 0.2 M Tris–HCl buffer (pH 8.0). The fractions containing glutathione *S*-transferase activity were concentrated with an ultrafiltration cell (model 8050, Amicon, Danvers, MA) and were chromatographed on a Sephadex G-100 column. Fractions with glutathione *S*-transferase activity were combined and concentrated to 6 ml by ultrafiltration and were then dialyzed against 300 ml of 0.01 M potassium phosphate buffer (pH 6.7) for 2 hr. The Sephadex G-100 fractions with glutathione *S*-transferase activity were chromatographed on a CM52 column and eluted with a linear gradient of potassium chloride (0–100 mM) in 0.01 M potassium phosphate buffer (pH 6.7). The nonadsorbed fractions from the CM52 columns were concentrated, diluted with an equal volume of starting buffer (0.01 M potassium phosphate, pH 6.7, containing 0.1 mM EDTA, 2 mM glutathione, and 30% glycerol), and applied to a hydroxylapatite column, which was eluted with a linear gradient of 0.01 to 0.3 M phosphate buffer (pH 6.7). Fractions (1.2 ml or 2.5 ml) were collected. A sample of each fraction was used for the measurement of glutathione *S*-transferase activity by the method of Habig *et al.* [11] with CDNB as substrate and for quantification of radioactivity with a liquid scintillation counter. When microsomal glutathione *S*-transferase activity was measured, 3 mM glutathione was used instead of 1 mM.

The protein content of column fractions was determined by measuring the absorbance at 280 nm, and the protein concentration of pooled fractions was measured by the method of Lowry *et al.* [12]; when protein concentrations were measured in the eluate from the hydroxylapatite column, glutathione or glycerol was removed by centrifugation with a microconcentrator (Centricon, Amicon, Danvers, MA).

SDS-Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [13] or Warner and Gorenstein [14]. Gels were dried and exposed to Kodak X-OMAT AR film for autoradiography.

RESULTS

Cytosolic glutathione *S*-transferase activity, with CDNB as the substrate, after incubation with or without [^{14}C]bromobenzene was 1.83 ± 0.12 and 1.87 ± 0.14 (mean ± SD, N = 3) μmol product/mg protein, respectively. The incubation mixtures containing [^{14}C]bromobenzene were centrifuged, and the cytosolic fraction was used for purification of glutathione *S*-transferases by column chromatography. Figure 1 shows a DE52 chromatographic profile of hepatic cytosol from a homogenate incubated with [^{14}C]bromobenzene. Significant glutathione *S*-transferase activity was present in fractions 5 through 15 (designated DE52-1), and some activity was present as a second peak in fractions 60 through 66 (DE52-3). Radioactivity was

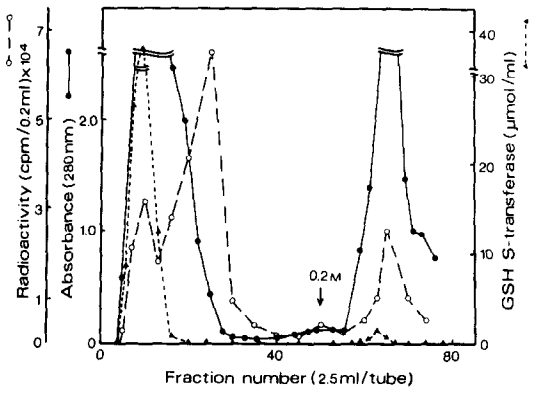

Fig. 1. DE52 column chromatography of cytosol incubated with [^{14}C]bromobenzene. Cytosol (284 mg) from an incubation mixture containing [^{14}C]bromobenzene was applied to a DE52 column (1.6 × 13 cm), which was eluted with 0.01 M Tris–HCl buffer (pH 8.0). At fraction 50, the elution buffer was changed to 0.2 M Tris–HCl buffer (pH 8.0). Glutathione *S*-transferase activity, protein concentrations, and content of radioactivity in the fractions were measured as described in Materials and Methods.

detected in those fractions with transferase activity and in the latter part of the first peak (fractions 17 to 31) (DE52-2), which showed no transferase activity but contained considerable radioactivity. Ninety-two percent of the glutathione *S*-transferase activity in cytosol was recovered in the fractions collectively designated as DE52-1. The radioactivity in this fraction represented 7.1% of the total present in the cytosol, whereas 4.3% of the radioactivity was present in fraction DE52-2. Twenty percent of radioactivity in DE52-1 and 99% of the radioactivity in DE52-2 were lost by ultrafiltration; thus, these data show that a portion of the radioactivity in DE52-1 and all of the radioactivity in DE52-2 represent unbound [^{14}C]bromobenzene or metabolites. The fraction designated DE52-1 was chromatographed on a Sephadex G-100 column (Fig. 2). Ninety-eight

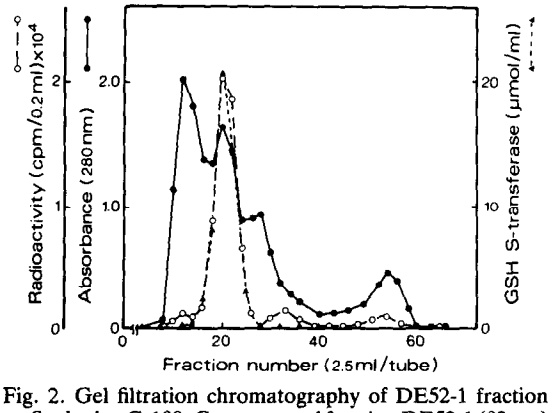

Fig. 2. Gel filtration chromatography of DE52-1 fraction on Sephadex G-100. Concentrated fraction DE52-1 (83 mg) was applied to a Sephadex G-100 column (1.6 × 85 cm), which was eluted with 0.01 M potassium phosphate buffer (pH 7.4). Glutathione *S*-transferase activity, protein concentrations, and content of radioactivity in the fractions were measured as described in Materials and Methods.

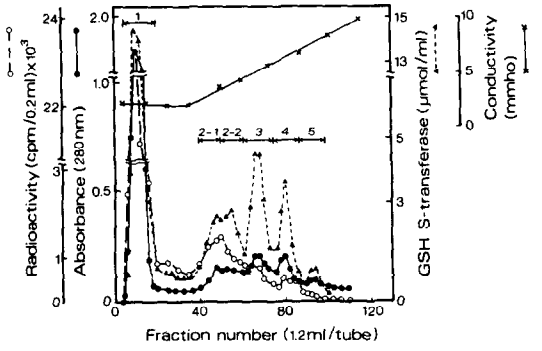

Fig. 3. CM52 column chromatography of Sephadex G-100 fractions. After concentration and dialysis, Sephadex G-100 fraction G-100-2 with glutathione *S*-transferase activity (30 mg) was applied to a CM52 column (0.9 × 21 cm), which was eluted with a linear gradient of potassium chloride (0–100 mM) in 0.01 M potassium phosphate buffer (pH 6.7). Peaks designed at 1, 2-1, 2-2, 3, 4, and 5 correspond to glutathione *S*-transferases 4-4/5-5, 1-1 or 3-4, 1-2, 3-3, and 2-2 respectively. Glutathione *S*-transferase activity, protein concentrations, conductivity, and content of radioactivity in the fractions were measured as described in Materials and Methods.

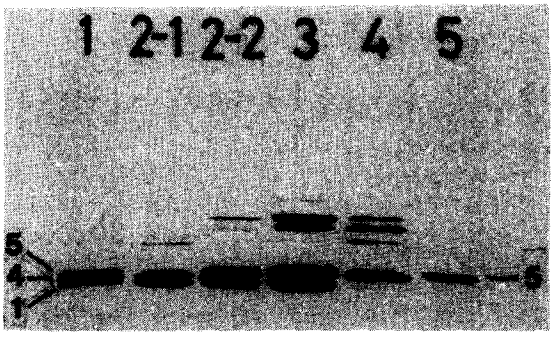

Fig. 4. SDS-Polyacrylamide gel electrophoresis of CM52 fractions. CM52 fractions isolated from bromobenzene-exposed liver homogenates (Fig. 3) were concentrated by ultrafiltration and were separated by SDS-polyacrylamide gel electrophoresis, as described in Materials and Methods. Lanes from left to right contained fractions CM52-1, CM52-2-1, CM52-2-2, CM52-3, CM52-4, and CM52-5. Bands labeled 5, 4, and 1 correspond to glutathione *S*-transferase subunits 5, 4 and 1 respectively.

percent of the glutathione *S*-transferase activity and 79% of the radioactivity applied to the column were detected in the second peak (fractions 16 to 26), which was designated G-100-2. The radioactivity in the fourth peak (fractions 50 to 60) appears to represent unbound [^{14}C]bromobenzene or metabolites.

Figure 3 shows the CM52 chromatographic profile of Sephadex fraction G-100-2. Six peaks with glutathione *S*-transferase activity were designated as 1, 2-1, 2-2, 3, 4, and 5 in order of elution from the CM52 column. Greatest radioactivity was present in peaks 1 (CM52-1), 2-1 (CM52-2-1), and 2-2 (CM52-2-2). To determine the subunit composition of the cytosolic glutathione *S*-transferases, SDS-polyacrylamide gel electrophoresis of each CM52 fraction was done, and the results are shown in Fig. 4. Peak CM52-1 apparently contained transferase subunits 1, 5, and 4.* The subunit content of peaks CM52-2-1, CM52-2-2, CM52-3, CM52-4, and CM52-5 appears to coincide with the reported subunit compositions

* Although it is recognized that the electrophoretic analysis would fail to distinguish subunit 4 from subunit 3, because both have the same molecular weight (M_r = 26,500) [10, 15], the elution of transferases in peak CM52-1 (Fig. 3) corresponds to the reported elution of transferases 4-4 and 5-5 from a CM52 column [11]. Hence, we conclude that transferase subunits with M_r = 28,500 and 26,500 (Fig. 7) correspond to transferase subunits 5 and 4 respectively.

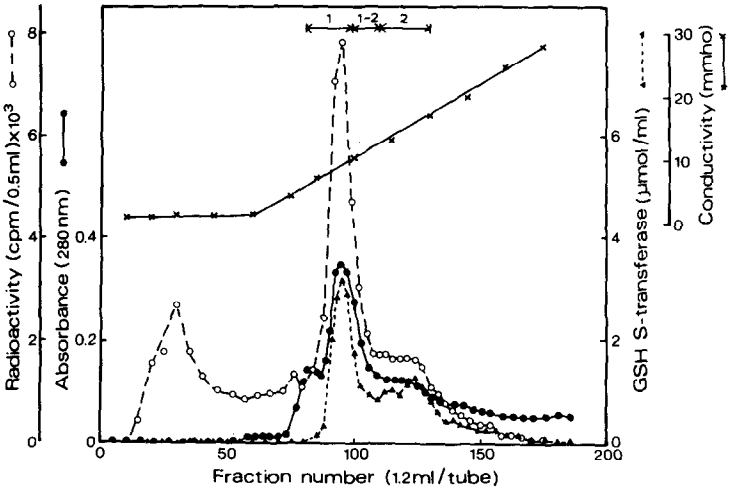

Fig. 5. Hydroxylapatite column chromatography of CM52 fractions. After concentration, fraction CM52-1 (9.0 mg) was diluted with an equal volume of the starting buffer and applied to a hydroxylapatite column (1.6 × 11 cm). The column was eluted with a linear gradient of 0.01 to 0.3 M potassium phosphate buffer (pH 6.7) containing 2 mM glutathione, 0.1 mM EDTA, and 30% glycerol. Glutathione *S*-transferase activity, protein concentrations, conductivity, and content of radioactivity in the fractions were measured as described in Materials and Methods.

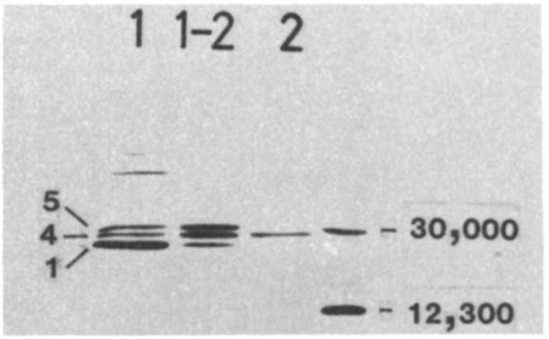

Fig. 6. SDS-Polyacrylamide gel electrophoresis of hydroxylapatite fractions. The fractions eluted from the hydroxylapatite column (Fig. 5) were concentrated by centrifugal ultrafiltration, and the protein was dissolved in water. A sample of each fraction was analyzed by electrophoresis (10% gel) [13]. The lanes contained 1 (fraction numbers 79–100), 1-2 (fraction numbers 101–110), and 3 (fraction numbers 111–130). The position of the molecular weight markers is shown: 30,000 (carbonic anhydrase) and 12,300 (cytochrome *c*). Bands labeled 5, 4, and 1 correspond to glutathione *S*-transferase subunits 5, 4, and 1 respectively.

of transferases 3-4, 3-4, 1-1 or 1-2, 3-3, and 2-2 respectively [10, 11]. Because peak CM52-1 contains much glutathione *S*-transferase activity and much subunit-associated radioactivity, further purification was done on a hydroxylapatite column. As shown in Fig. 5, radioactivity was detected in three peaks around fractions 15 to 40, 79 to 100, and 111 to 130. Protein was not detected in the first peak, and the radioactivity present in this peak appears to represent unbound [^{14}C]bromobenzene and metabolites. The second peak contained considerable radioactivity and much glutathione *S*-transferase activity, whereas both radioactivity and enzyme activity were low in the third peak. Analysis of the transferase subunit content by SDS-polyacrylamide gel electrophoresis (Fig. 6) showed that fractions 1 and 1-2 (Fig. 5) apparently contained subunits 1, 5, and 4, and the third fraction (designated 2, Fig. 5) contained primarily subunit 4. Fractions 1 and 1-2 (Fig. 5) contained the majority of subunit 1, which was not identified conclusively as ligandin (transferase 1-1 or 1-2).

To determine whether radioactivity derived from [^{14}C]bromobenzene was preferentially associated with the individual glutathione *S*-transferase subunits, hydroxylapatite fractions 1-2 and 2 (Fig. 5) were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. As shown in Fig. 7, [^{14}C]bromobenzene-derived radioactivity was apparently associated with glutathione *S*-transferase subunit 1 and, to a lesser extent, subunit 4. No [^{14}C]bromobenzene-derived radioactivity was associated with glutathione *S*-transferase subunit 5 (Fig. 7).

The recoveries of protein, transferase activity, and radioactivity are summarized in Table 1. Radioactivity, on a cpm per mg protein basis, was pre-

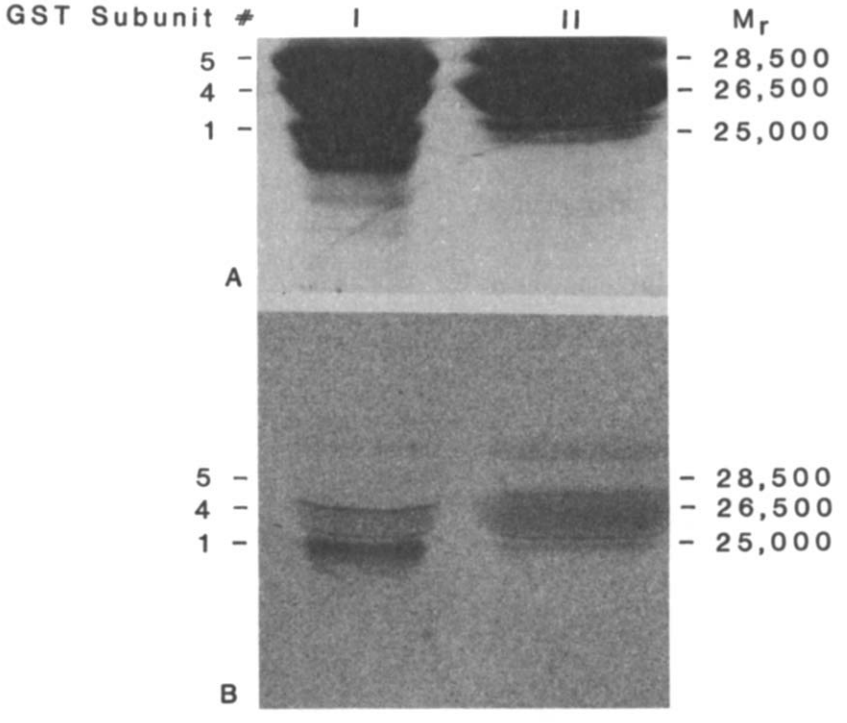

Fig. 7. SDS-Polyacrylamide gel electrophoresis of purified glutathione *S*-transferases. (A) A 15% polyacrylamide gel [14] of pooled hydroxylapatite fractions 1-2 and 2 (Fig. 5) is shown. Lanes I and II contained 25 μg protein from fractions 1-2 and 2 respectively. The indicated M_r values of the glutathione *S*-transferase subunits were determined with low-molecular weight protein standards. (B) Autoradiogram of the gel in A (above) indicates association of [^{14}C]bromobenzene-derived radioactivity with glutathione *S*-transferase subunits 1 and 4.

Table 1. Recovery of protein, glutathione S-transferase (GST) activity, and radioactivity from chromatography of cytosolic and column fractions

Step	Volume (ml)	Protein			GST activity				Radioactivity		
		mg/ml	mg	%	μmol/ml	μmol/mg	μmol	%	cpm × 10^{-3}	%	cpm × 10^{-3}/mg
Cytosol	15.0	19.0	284.4	100	34.1	1.8	511.2	100	31,800	100	
DE52-1	24.0	4.2	100.1	35.2	19.7	4.7	472.3	92.4	2,260	7.1	
conc-DE52-1	9.0	9.3	83.4	29.3	43.0	4.6	386.6	75.6	1,890	6.0	
G-100-2	29.0	1.0	30.4	10.7	13.1	12.4	379.0	74.1	1,490	4.7	49.1
CM52-1	17.0	0.6	10.9	3.8	7.6	11.9	129.2	25.3	838	2.6	76.9
2-1	16.5	0.1	1.1	0.4	2.0	31.2	33.0	6.5	92.2	0.3	83.9
2-2	12.5	0.1	1.4	0.5	2.3	20.2	28.6	5.6	70.1	0.2	50.1
3	17.5	0.1	2.4	0.8	2.7	20.0	47.4	9.3	59.0	0.2	24.6
4	12.0	0.1	1.4	0.5	2.2	19.3	26.4	5.2	25.5	0.1	18.2
5	12.0	0.1	0.6	0.2	0.7	13.8	5.2	1.7	7.02	0.02	11.7
conc-CM52-1	6.8	1.3	9.0	3.2	16.4	12.3	111.6	21.8	587	1.8	64.9
Hydroxyl-0'	27.0								89.6	0.3	
1	22.0	0.1	2.6	0.9	1.32	11.2	29.1	5.7	160	0.5	61.6
1-2	8.0	0.1	1.0	0.3	1.13	9.0	9.1	1.8	39.9	0.1	39.9
2	12.5	0.1	0.8	0.3	1.07	16.7	13.4	2.6	41.4	0.1	51.8

The recoveries of protein, glutathione S-transferase (GST) activity and radioactivity, which were measured as described in Materials and Methods, from cytosol and from DE52 (Fig. 1), Sephadex G-100 (Fig. 2), CM52 (Fig. 3), and hydroxylapatite (Fig. 5) columns are shown.

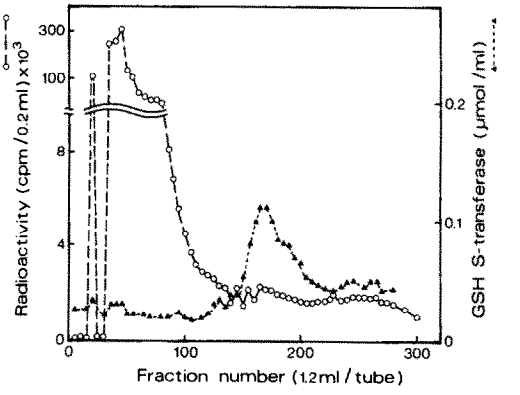

Fig. 8. Separation of microsomal glutathione S-transferases on a hydroxylapatite column. The microsomal fractions (98 mg), which were prepared as described in Materials and Methods, were homogenized with 3 ml of 0.025 M sucrose and, after the addition of 8 ml of the elution buffer, were applied to a hydroxylapatite column (1.6 × 13 cm), which was eluted with a linear gradient of 0.01 to 0.3 M potassium phosphate buffer (pH 7.0) containing 1 mM glutathione, 0.1 mM EDTA, 1% Triton X-100, and 20% glycerol. Glutathione S-transferase activity and the content of radioactivity in the fractions were measured as described in Materials and Methods.

dominant in fractions CM52-1, CM52-2-1, and CM52-2-2, and the glutathione S-transferase activities of these fractions were 11.9, 31.2, and 20.0 μmol product/mg protein respectively. Cytosolic glutathione S-transferases were purified from control rats, and the CM52 chromatographic profile was similar to that found in homogenates incubated with bromobenzene, except that the protein peak of fraction CM52-1 was smaller and fraction CM52-2-2 was larger than that seen in homogenates incubated with bromobenzene (data not shown). The enzyme activities of CM52 fractions 1 and 2 in the controls were 6–8 and 22–28 μmol product/mg protein respectively. These results show that bromobenzene increases, rather than decreases, the transferase activity in fraction CM52-1. The transferase activities in other CM52 fractions from the control homogenates did not differ from those observed in homogenates incubated with bromobenzene.

The microsomal glutathione S-transferases, which were isolated from homogenates incubated with [^{14}C]bromobenzene, were separated on a hydroxylapatite column (Fig. 8). The largest amount of radioactivity was detected in nonadsorbed fractions (fraction number 20 to 100), and the fractions (150 to 200) with glutathione S-transferase activity did not contain appreciable radioactivity.

DISCUSSION

Bromobenzene causes centrilobular hepatic necrosis in rats, which is associated with the formation of reactive metabolites that covalently bind to liver macromolecules [1–4]. The major reactive metabolite of bromobenzene is bromobenzene-3,4-oxide [5, 6], which is conjugated with glutathione by the glutathione S-transferases and is bound covalently to cellular macromolecules after depletion of

glutathione [3–5]. Therefore, the glutathione *S*-transferases play an important role in the detoxification of bromobenzene metabolites.

Previous studies have shown that treatment of rats with several hepatotoxic chemicals alters both the chromatographic behavior of cytosolic glutathione *S*-transferases and the catalytic activity of the more basic glutathione *S*-transferases [16, 17]. These findings were extended in the present study. It was demonstrated that the glutathione *S*-transferase activity with CDNB as the substrate in liver homogenates was not decreased after incubation with [^{14}C]bromobenzene and that radioactivity derived from [^{14}C]bromobenzene eluted with glutathione *S*-transferase activity when the cytosol was chromatographed on a DE52 column (Fig. 1). Moreover, further purification of the transferases showed that the radioactivity was preferentially associated with fraction CM52-1 and, to a lesser extent, with fractions CM52-2-1 and CM52-2-2 (Fig. 3). Glutathione *S*-transferases 4-4 and 5-5 normally elute first from a CM52 column (Fig. 3 and Refs. 11 and 17). Bromobenzene treatment of rats perturbs the CM52 chromatographic profile of the glutathione *S*-transferases so that the activity normally associated with transferases 1-1, 1-2, 2-2, 3-3, and 3-4 elutes with transferases 4-4 and 5-5 [17]. Presumably a similar phenomenon occurred in the present *in vitro* experiments. This may serve to explain the apparent presence of subunits not normally associated with transferases 4-4 and 5-5 in fraction CM52-1; hence, electrophoretic analysis indicates the presence of subunit 1 where only subunits 4 and 5 would be expected (Figs. 6 and 7). Moreover, [^{14}C]bromobenzene-derived radioactivity was associated primarily with subunit 1 of fraction CM52-1. Sheehan and Mantle [18] reported the presence of two populations of transferase subunit 1 with different kinetic properties but with the same molecular weight; one eluted before glutathione *S*-transferases 3-4 from a CM cellulose column at pH 6.7, and the other eluted at pH 6.0. Presumably both subunits were present in the CM52-1 and hydroxylapatite fractions in the present experiment. Because the radioactivity remained associated with the protein after gel filtration, ion-exchange chromatography, ultrafiltration, and dialysis during the purification of the cytosolic glutathione *S*-transferases, the radioactivity associated with the transferases appears to be irreversibly bound [^{14}C]bromobenzene or metabolites.

Furthermore, it was demonstrated that the glutathione *S*-transferases retained their catalytic activities with CDNB as the substrate, although the proteins had been arylated by bromobenzene metabolites. This is consistent with the finding that glutathione *S*-transferases possess nonsubstrate ligand-binding sites as well as catalytic sites. The former may be blocked by covalent labeling with bilirubin-Woodward's reagent K but the catalytic activity is not inhibited [19]. It is, therefore, assumed that bromobenzene metabolites bind covalently to the nonsubstrate ligand-binding site of subunit 1 but not to the catalytic site. Thus, these data indicate that the glutathione *S*-transferases may contribute to detoxication of bromobenzene reactive metabolites by serving as trapping proteins that scavenge reactive metabolites.

Monks *et al.* [9] also showed that bromobenzene metabolites become covalently bound to glutathione *S*-transferases. Moreover, ethacrynic acid [20], paracetamol [21], and chloroform metabolites (Y. Aniya and M. W. Anders, unpublished observations) all become covalently bound to the glutathione *S*-transferases. These reports and the observations reported in this paper strengthen the concept [22] that the transferases serve at least three important detoxication functions: (1) the glutathione *S*-transferases catalyze the reaction of electrophilic compounds or metabolites with glutathione to yield premercapturic acids, which are metabolized to mercapturic acids that are readily excreted; (2) the glutathione *S*-transferases have important roles as storage and transport proteins; and (3) the glutathione *S*-transferases detoxify electrophilic metabolites by serving as targets for alkylation or arylation. And, as shown in the present study, there may be considerable subunit selectivity in the arylation of the glutathione *S*-transferases, which suggests that the arylation reaction is a directed, rather than random, event. Further studies on the mechanism and biological significance of the alkylation and arylation of catalytic proteins by reactive metabolites are warranted.

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